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Isolation, pH-Optima and Apparent *Michaelis* Constants of Highly Purified Enzymes from Human and Animal Sources

Comparison of Enzymes of Human and Animal Origin, I

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Summary: Enzymes from animal sources are frequently used for quality control of enzyme activity determinations in clinical chemistry. For this purpose they should be very similar to human enzymes. It is shown that preparations of enzymes of diagnostic interest from human and porcine organs, purified in exactly the same way, have very similar pH-optima and apparent *Michaelis* constants for their substrates.

Isolierung, pH-Optima und apparente Michaelis-Konstanten hoch gereinigter Enzyme aus humanem und tierischem Gewebe.

Vergleich humaner und tierischer Enzyme, I. Mitteilung

Zusammenfassung: Enzympräparate tierischen Ursprungs werden häufig für den Einsatz in Qualitätskontrollproben für die klinische Chemie verwendet. Zu diesem Zweck sollten sie humanen Enzymen möglichst ähnlich sein. Es wird gezeigt, daß Enzyme von diagnostischem Interesse, welche aus Human- und aus Schweine-Organen auf gleiche Weise hoch gereinigt wurden, sehr ähnliche pH-Optima und apparente *Michaelis*-Konstanten gegenüber ihren Substraten aufweisen.

Introduction

For quality control in determinations of the activity of enzymes in Clinical Chemistry materials with enhanced enzyme content are frequently needed. Patients' sera with an endogenous enhancement of these enzymes are not available in sufficient quantities and may show stability problems, especially on shipment for interlaboratory surveys. Materials, to which enzyme preparations have been added, are preferable for routine use. These enzymes should be as closely related to those in the patients' sera as possible in any aspect of routine analytical importance. Therefore, isolation of such enzymes from human organs would be a good choice, but is restricted by ethical reasons. Only two human organs are easily available, blood or blood fractions and placenta. Enzyme preparations useful for quality control and standardization purposes, have been obtained from both these sources, e. g. aspartate aminotransferase (*L*-Aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) (1) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) (2). But most of the enzymes needed cannot be adequately prepared from these materials.

It is therefore the intention of this study and of two following papers (3, 4) to recheck dissimilarities of analytical interest between human and mammalian enzymes. Preliminary reports on this work were given at the 1st European Congress on Clinical Chemistry in Munich 1974 (5, 6) and at the 9th International Congress of Clinical Chemistry in Toronto 1975 (7).

Nowdays, all enzymes of diagnostic interest are extensively studied with respect to their chemical, catalytic and physical properties. The data are accumulated in different hand-books, e. g. (8, 9, 10, 11). Also, there is much published data on the comparison of enzymes and isoenzymes (12, 13) from different animal sources, including more limited information on enzymes from human organs. Some dissimilarities in catalytic properties, which are apparent from different publications may be due mainly to differences in the isolation procedures, the state of purity and the assay conditions and not to

basic differences in catalytic properties. We therefore decided to isolate these enzymes from the same organs, using the same isolation steps and to perform the assays simultaneously in exactly the same assay media.

The characteristics of the isolated enzymes that were used for comparison were pH-optima and apparent *Michaelis* constants. As cited e. g. in l. c. (14) apparent *Michaelis* constants of multi component reactions depend on the assay conditions used (e. g. temperature, substrate concentrations, salt content, activators, inhibitors, pH, etc.). We therefore chose methodologies of broader international use. It should be noted that the values given are only found for these special conditions, whereas our main concern is to show those similarities found between the human and animal enzymes, which should also be valid for other assay media.

Materials and Methods

All enzymes, coenzymes and most substrates used were products of Boehringer Mannheim GmbH. Determinations of enzyme activities were performed with Test-Combinations of Boehringer Mannheim GmbH, where appropriate. Buffers and other reagents were purchased from E. Merck, Darmstadt.

The MM isoenzyme of *creatine kinase* (ATP: creatine N phosphotransferase, EC 2.7.3.2) was prepared according to *Keutel* et al. (15) from rabbit and pig muscle and from human psoas muscle. The BB isoenzyme of creatine kinase was isolated in crystalline form from pig and human brain according to *Atherton* et al. (16).

γ-glutamyl transferase ((γ-glutamyl)-peptide: amino-acid γ-glutamyl-transferase, EC 2.3.2.2) was purified according to *Orlowski & Meister* (17) using the modification of *Richter* (18) from human and porcine kidneys.

Aspartate aminotransferase (*L*-Aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1), *alanine aminotransferase* (*L*-Alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2), H_4-isoenzyme of *lactate dehydrogenase* (*L*-Lactate: NAD oxidoreductase, EC 1.1.1.27) and *malate dehydrogenase* (*L*-Malate: NAD oxidoreductase, EC 1.1.1.37) were separated from each other and partially purified from human and porcine heart muscle in a procedure related to the work of *Beisenherz* et al. (19) on the purification of rabbit muscle enzymes.

Aspartate aminotransferase (the cytoplasmic s-enzyme) and alanine aminotransferase were further purified according to (20, 21).

For final purification of H_4-isoenzyme of lactate dehydrogenase and malate dehydrogenase we used methods as published by *Beisenherz* et al. (19) and by *Glatthaar* et al. (22) respectively.

M_4 isoenzyme of lactate dehydrogenase from human and porcine muscle was crystallized according to (19).

The M_4 and H_4 isoenzymes of lactate dehydrogenase were separated from the other isoenzymes by chromatography using DEAE cellulose; they each showed single bands in disk electrophoresis.

Glutamate dehydrogenase (*L*-Glutamate: NAD (P) oxidoreductase, EC 1.4.1.3) from human and ox liver was crystallized according to *Lehmann & Pfleiderer* (23).

The specific activities of the purified enzymes are summarized in table 1.

Enzyme activity assays were performed using an Eppendorf-photometer, Model 1101 M, with automated cuvette changing and scanning device. Wavelengths 365 nm and 405 nm; temperature 25 °C.

For *protein determinations* we used the biuret method (as cited in l. c. 8). To get correct protein values for γ-glutamyl transferase it is necessary to complete the precipitation with trichloracetic acid by heating (5 min, 95 °C).

The *initial reaction velocity values* for the determination of the apparent *Michaelis* constants were obtained by starting the reaction with appropriate enzyme dilutions, using substrate concentrations in the suitable range in 1:2, 1:4, 1:8 etc. dilutions. The assay conditions are stated below. The apparent K_m-values were derived from *Lineweaver-Burk* plots (24).

The *optimum pH-values* were determined with the assay conditions described below. The pH-readings were taken in the cuvette immediately following the enzyme activity assay. All assays for the same enzymes from different origins were performed in series, by the same welltrained technician, using the same instrumentation and reagents.

Results and Discussion

General Remarks

Purification

As can be seen from table 1 the final purity of the same enzyme from different origins as expressed in specific activity is in the same order of magnitude for all enzymes studied. The same was true for the yield. With all of these enzymes there was no problem in adapting the purification procedures well-known to us for the animals enzymes to the isolation of the human ones: any purification step resulted in material of similar specific activity in adequate yield. It may be concluded that known differences in the protein structure of the enzymes from different origins (to be discussed in l. c. (3)) are not important enough to interfere in the purification procedures used.

Stability

All the enzyme preparations have now been stored at 4 °C for 2.5 years. (Aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase (H_4- and M_4-isoenzymes) and malate dehydrogenase as suspensions in 3.2 mol/l ammonium sulphate solution; glutamate dehydrogenase as a solution in 50% aqueous glycerol; creatine kinase and γ-glutamyl transferase as saltfree lyophylates, γ-glutamyl transferase containing some Triton X 100). The stability of the same enzyme from different origins was shown to be of same magnitude again, indicating further similarities of theoretical and practical importance.

Creatine Kinase

The pH-optima and apparent K_m-values of the MM isoenzyme of creatine kinase from rabbit, porcine and human psoas muscle are summarized in table 2; those of the BB isoenzymes from the different brains are in table 3.

For the determination of the creatine phosphorylation we used conditions according to *Tanzer & Gilvarg* (25), and for the reverse reaction we used those given by *Forster* et al. (26). The conformity of figures for the

Tab. 1. Comparison of the specific activity of the enzymes isolated from human and mammalian organs.

Enzyme	Source	U/mg protein (25 °C)	Assay conditions (8)
creatine kinase MM	rabbit muscle	250	triethanolamine buffer 0.1 mol/l, pH 7.0;
MM	human psoas muscle	315	substrates:
MM	porcine muscle	251	creatine phosphate and ADP,
BB	human brain	286	activation by glutathione,
BB	porcine brain	328	continuous photometric registration at 365 nm.
γ-glutamyl transferase	human kidneys	20	Tris-buffer 185 mmol/l,
	porcine kidneys	20	glycylglycine 40 mmol/l, pH 8.25;
			γ-glutamyl-4'-nitranilide 4 mmol/l,
			continuous photometric registration at 405 nm.
aspartate aminotransferase	human heart	210	phosphate buffer 80 mmol/l, pH 7.4,
	porcine heart	200	substrates:
			aspartate and 2-oxoglutarate,
			continuous photometric registration at 365 nm.
alanine aminotransferase	human heart	78	phosphate buffer 80 mmol/l, pH 7.4,
	porcine heart	80	substrates:
			L-alanine and 2-oxoglutarate,
			continuous photometric registration at 365 nm.
glutamate dehydrogenase	human liver	250	imidazole buffer 85 mmol/l, pH 7.9,
	bovine liver	190	substrates:
			2-oxoglutarate and ammonia,
			activation by ADP 2 mmol/l,
			continuous photometric registration at 365 nm.
lactate dehydrogenase H_4	human heart	375	phosphate buffer 0,1 mol/l, pH 7.5,
H_4	porcine heart	360	substrate:
M_4	human psoas muscle	395	pyruvate,
M_4	porcine muscle	450	continuous photometric registration at 365 nm.
malate dehydrogenase	human heart (cytoplasmic)	1060	phosphate buffer 0,1 mol/l, pH 7.5,
	porcine heart (cytoplasmic)	1100	substrate:
			oxaloacetate,
			continuous photometric registration at 365 nm.

same enzyme from different sources is sufficient, taking into account the relatively low precision of the three-fold coupled reaction (C. V. approx. 8 % in the range studied) and a slight instability of the enzyme in the very dilute solutions used (physiological sodium chloride solution, no protein or thiol additives). Comparability of apparent K_m-values can only be reached by using exactly the same reaction conditions, as the two substrates show an interdependency in both reaction directions; there also occur product and salt inhibition, dependency on magnesium ion versus nucleotide concentration ratios and so on. These were pointed out for the MM isoenzyme (of rabbit muscle) by *Morrison* et al. (27) and for the BB isoenzyme by *Jacobs* et al. (28) and discussed by *Watts* (29). But by calculation from their data (assayed at 30 °C) one can derive figures for apparent K_m at similar concentrations quite close to those stated here, e. g. for rabbit MM isoenzyme of creatine kinase (27):

creatine phosphate	4.2 mmol/l
adenosine-5'-diphosphate	0.57 mmol/l
creatine	9.4 mmol/l
adenosine-5'-triphosphate	0.59 mmol/l

Data published by *Szasz* et al. (30) recently for apparent K_m-values for optimized conditions for creatine kinase activity measurement in human sera differ considerably, due to important differences in most of the reaction parameters.

γ-Glutamyl Transpeptidase

γ-glutamyl transpeptidase preparations from kidneys tend to aggregate in solutions not containing detergents (31), giving rise to turbidities and loss of activity of the soluble part. The data on pH-optima and apparent *Michaelis* constants were therefore derived using enzyme dilutions in Tris-buffer, 20 mmol/l, pH 8.25, containing 0,001 % Triton X 100.

As shown in table 4 values for the human and porcine preparations were absolutely identical.

The reaction conditions used were according to *Szasz* (32). It is therefore not very surprising that the apparent K_m of 0.96 mmol/l for *L*-γ-glutamyl-*p*-nitranilide as determined by *Szasz* in patient sera is exactly the same as ours. But these findings indicate catalytic similarities

Tab. 2. Creatine kinase from muscle (CK-MM) – Apparent *Michaelis* constants and pH-optima – at 25 °C.

	Rabbit muscle	Porcine muscle	Human psoas muscle	Assay conditions (Concentrations of constituents in the assay solution) (8)
Optimum pH Substrate: Creatine	8.5	8.8	8.5	glycine 0.45 mol/l; creatine 32 mmol/l; ATP 1.2 mmol/l; NADH 0.2 mmol/l; phosphoenol-pyruvate 0.4 mmol/l; M_4 isoenzyme of lactate dehydrogenase 28.5 mg/l ⩾ 14 kU/l; pyruvate kinase 28.5 mg/l ⩾ 5.7 kU/l; $MgCl_2$ 7 mmol/l.
Optimum pH Substrate: Creatine Phosphate	6.5	7.0	6.5	triethanolamine 0.1 mol/l; glucose 20 mmol/l; Mg-acetate 10 mmol/l; ADP 2 mmol/l; creatine-phosphate 34 mmol/l; AMP 10 mmol/l; NADP 0.6 mmol/l; glutathione 10 mmol/l; hexokinase 7.4 mg/l ⩾ 1.0 kU/l; glucose-6-phosphate-dehydrogenase 7.4 mg/l ⩾ 1.0 kU/l.
App. K_m Creatine	11 mmol/l	11 mmol/l	15 mmol/l	glycine 0.45 mol/l, pH 9.0; ATP 1.2 mmol/l; NADH 0.2 mmol/l; phosphoenol-pyruvate 0.4 mmol/l; $MgCl_2$ 7 mmol/l; pyruvate kinase 28.5 mg/l ⩾ 5.7 kU/l; M_4 isoenzyme of lactate dehydrogenase 28.5 mg/l ⩾ 14 kU/l.
App. K_m ATP	480 µmol/l	330 µmol/l	440 µmol/l	glycine 0.45 mol/l, pH 9.0; creatine 32 mmol/l; NADH 0.2 mmol/l; phosphoenol-pyruvate 0.4 mmol/l; $MgCl_2$ 7 mmol/l; pyruvate kinase 28.5 mg/l ⩾ 5.7 kU/l; M_4 isoenzyme of lactate dehydrogenase 28.5 mg/l ⩾ 14 kU/l.
App. K_m Creatine Phosphate	7.7 mmol/l	7.1 mmol/l	10 mmol/l	triethanolamine 0.1 mol/l, pH 7.0; glucose 20 mmol/l; magnesium acetate 10 mmol/l; ADP 2 mmol/l; AMP 10 mmol/l; NADP 0.6 mmol/l; glutathione 10 mmol/l; hexokinase 7.4 mg/l ⩾ 1.0 kU/l; glucose-6-phosphate-dehydrogenase 7.4 mg/l ⩾ 1.0 kU/l.
App. K_m ADP	360 µmol/l	330 µmol/l	500 µmol/l	triethanolamine 0.1 mol/l, pH 7.0; glucose 20 mmol/l; magnesium acetate 10 mmol/l; creatine phosphate 34 mmol/l; AMP 10 mmol/l; NADP 0.6 mmol/l; glutathione 10 mmol/l; hexokinase 7.4 mg/l ⩾ 1.0 kU/l; glucose-6-phosphate-dehydrogenase 7.4 mg/l ⩾ 1.0 kU/l.

between the purified enzymes from kidney and the liver enzyme, which is mainly present in patients' sera.

As the reaction mechanism of γ-glutamyl transferase has been much less studied than that of the other enzymes, the dependency of the apparent K_m-values on different reaction conditions is not quite clear. *Richter* (33) found the K_m for *L*-γ-glutamyl-*p*-nitranilide to be 5.8 mmol/l at 37 °C using tris-buffer, pH 9.0; *Miller* et al. (31) defined a value of 0.8 mmol/l, (37 °C, ammediol-buffer, pH 8.5.) The latter authors discuss possible differences between the human and the hog kidney enzyme, which were not found in the present study.

Aspartate Aminotransferase

The figures shown in table 5 for pH-optima and apparent *Michaelis* constants for cytoplasmic s-enzyme from human and porcine heart muscle are totally identical. (The small discrepancy with 2-oxoglutarate is within the experimental limits of error, CV 5 %). The substrate concentrations chosen in the most commonly used routine detection of oxaloacetate produced by malate dehydrogenase coupling were similar to those of *Karmen* et al. (34). For the assays in the opposite reaction we detected the reaction product 2-oxoglutarate under appropriate conditions. As shown in recent publications the reaction mechanism of aspartate aminotransferase is of the "ping-pong bi bi"-type (35) with each substrate influencing the apparent K_m of the paired one in a mathematically distinct way (36, 37, 38). The dependency of K_m-values on small alterations in concentration of the second substrate is most important under suboptimized conditions as used in this study. The equalty of values shown for the preparations from different sources is therefore quite indicative of their catalytic similarity.

Tab. 3. Creatine kinase from brain (CK-BB) – Apparent K_m-values and pH-optima – at 25 °C.

	Porcine brain	Human brain	Assay conditions (Concentrations of constituents in the assay solution) (8)
Optimum pH Substrate: Creatine	9.0	8.9	glycine 0.45 mol/l; creatine 32 mmol/l; ATP 1.2 mmol/l; NADH 0.2 mmol/l; phosphoenol-pyruvate 0.4 mmol/l; M_4 isoenzyme of lactate dehydrogenase 28.5 mg/l ≥ 14 kU/l; pyruvate kinase 28.5 mg/l ≥ 5.7 kU/l; $MgCl_2$ 7 mmol/l.
Optimum pH Substrate: Creatine Phosphate	6.7	6.7	triethanolamine 0.1 mol/l; glucose 20 mmol/l; magnesium-acetate 10 mmol/l; ADP 2 mmol/l; creatine phosphate 34 mmol/l; AMP 10 mmol/l; NADP 0.6 mmol/l; glutathione 10 mmol/l; hexokinase 7.4 mg/l ≥ 1.0 kU/l; glucose-6-phosphate-dehydrogenase 7.4 mg/l ≥ 1.0 kU/l.
App. K_m Creatine	1.4 mmol/l	1.5 mmol/l	glycine 0.45 mol/l, pH 9.0; ATP 1.2 mmol/l; NADH 0.2 mmol/l; phosphoenol-pyruvate 0.4 mmol/l; $MgCl_2$ 7 mmol/l; pyruvate kinase 28.5 mg/l ≥ 5.7 kU/l; M_4 isoenzyme of lactate dehydrogenase 28.5 mg/l ≥ 14 kU/l.
App. K_m ATP	140 µmol/l	190 µmol/l	glycine 0.45 mol/l, pH 9.0; creatine 32 mmol/l; NADH 0.2 mmol/l; phosphoenol-pyruvate 0.4 mmol/l; $MgCl_2$ 7 mmol/l; pyruvate kinase 28.5 mg/l ≥ 5.7 kU/l; M_4 isoenzyme of lactate dehydrogenase 28.5 mg/l ≥ 14 kU/l.
App. K_m Creatine phosphate	4.0 mmol/l	5.7 mmol/l	triethanolamine 0.1 mol/l, pH 7.0; glucose 20 mmol/l; magnesium acetate 10 mmol/l; ADP 2 mmol/l; AMP 10 mmol/l; NADP 0.6 mmol/l; glutathione 10 mmol/l; hexokinase 7.4 mg/l ≥ 1.0 kU/l; glucose-6-phosphate-dehydrogenase 7.4 mg/l ≥ 1.0 kU/l.
App. K_m ADP	300 µmol/l	300 µmol/l	triethanolamine 0.1 mol/l, pH 7.0; glucose 20 mmol/l; magnesium acetate 10 mmol/l; creatine phosphate 34 mmol/l; AMP 10 mmol/l; NADP 0.6 mmol/l; glutathione 10 mmol/l; hexokinase 7.4 mg/l ≥ 1.0 kU/l; glucose-6-phosphate-dehydrogenase 7.4 mg/l ≥ 1.0 kU/l.

Tab. 4. γ-Glutamyl-transpeptidase from kidneys – Apparent K_m-values and pH optima – at 25 °C.

	Porcine kidney	Human kidney	Assay conditions (Concentrations of constituents in the assay solution) (8)
Optimum pH	8.2	8.2	Tris-buffer 0.2 mol/l; γ-glutamy[illegible]nitranilide 4 mmol/l; glycylglycine 40 mmol/l.
App. K_m γ-glutamyl-*p*-nitroanilide	1.0 mmol/l	1.0 mmol/l	Tris-buffer, pH 8.25, 0.2 mol/l, [illegible]
App. K_m glycyl-glycine	33 mmol/l	33 mmol/l	Tris-buffer, pH 8.25, 0.2 mol/l; γ-glutamyl-*p*-nitroanilide 4 mmol/l.

In addition we checked the reactivation of both aspartate aminotransferase preparations by pyridoxal phosphate. The coenzyme can be completely removed in the pyridoxamine form from the apoenzyme by incubation with aspartate followed by dialysis at pH 5 (39). Both resulting apoenzyme preparations had zero transamination activity. Upon incubation with pyridoxal phosphate activity was fully restored (95–100% of original) within 1 min. Even in this respect the human and porcine enzyme behaved completely similarly.

Similar equivalence between highly purified aspartate aminotransferase from human erythrocytes and our pig heart preparation were reported by *Rej* (37), who also gave figures for true K_m-values for human m- and s-enzyme.

Alanine Aminotransferase

Table 6 shows the correspondence between pH-optima and apparent K_m-values for alanine aminotransferase preparations from pig and human heart muscle, as was found for the aspartate aminotransferase preparations.

Conditions chosen for the routinely used direction with detection of the pyruvate produced were again sub-

Tab. 5. Aspartate aminotransferase (cytoplasmic) from heart – Apparent K_m-values and pH-optima – at 25 °C.

	Pig heart	Human heart	Assay conditions (Concentrations of constituents in the assay solution) (8)
Optimum pH Substrate: aspartate	8.0–8.5	8.0–8.3	phosphate buffer 81 mmol/l; *L*-aspartate 32.5 mmol/l; 2-oxoglutarate 6.75 mmol/l; NADH 0.16 mmol/l; M_4 isoenzyme of lactate dehydrogenase 4.15 mg/l ⩾ 2.08 kU/l; malate dehydrogenase 4.15 mg/l ⩾ 4.6 kU/l.
Optimum pH Substrate: glutamate	7.1	7.1	triethanolamine 90 mmol/l; *L*-glutamate 50 mmol/l; oxaloacetate 1 mmol/l; NADH 0.2 mmol/l; ammonium acetate 106 mmol/l; glutamate dehydrogenase 67 mg/l ⩾ 6.0 kU/l.
App. K_m *L*-aspartate	6.2 mmol/l	6.2 mmol/l	phosphate buffer pH 7.4, 81 mmol/l; 2-oxoglutarate 6.75 mmol/l; NADH 0.16 mmol/l; M_4 isoenzyme of lactate dehydrogenase 4.15 mg/l ⩾ 2.08 kU/l; malate dehydrogenase 4.15 mg/l ⩾ 4.6 kU/l.
App. K_m 2-oxoglutarate	400 µmol/l	500 µmol/l	phosphate buffer pH 7.4, 81 mmol/l; *L*-aspartate 32.5 mmol/l; NADH 0.16 mmol/l; M_4 isoenzyme of lactate dehydrogenase 4.15 mg/l ⩾ 2.08 kU/l; malate dehydrogenase 4.15 mg/l ⩾ 4.6 kU/l.
App. K_m oxaloacetate	91 µmol/l	91 µmol/l	triethanolamine pH 8.0, 87 mmol/l; NADH 0.2 mmol/l; *L*-glutamate 48 mmol/l; ammonium acetate 106 mmol/l; glutamate dehydrogenase 333 mg/l ⩾ 30 kU/l.
App. K_m *L*-glutamate	6.7 mmol/l	6.7 mmol/l	triethanolamine pH 8.0, 87 mmol/l; oxaloacetate 2.6 mmol/l; NADH 0.2 mmol/l; ammonium acetate 106 mmol/l; glutamate dehydrogenase 333 mg/l ⩾ 30 kU/l.

Tab. 6. Alanine aminotransferase from heart – Apparent K_m-values and pH optima – at 25 °C.

	Porcine heart	Human heart	Assay conditions (Concentrations of constituents in the assay solution) (8)
Optimum pH Substrate: alanine	7.9–8.1	7.9–8.1	phosphate buffer 81 mmol/l; *D*, *L*-alanine 65 mmol/l; NADH 0.16 mmol/l; 2-oxoglutarate 6.75 mmol/l; M_4 isoenzyme of lactate dehydrogenase 4.15 mg/l ⩾ 2.08 kU/l.
Optimum pH Substrate: glutamate	7.6	7.6	triethanolamine 90 mmol/l; *L*-glutamate 50 mmol/l; pyruvate 2.2 mmol/l; NADH 0.2 mmol/l; ammonium acetate 106 mmol/l; glutamate dehydrogenase 67 mg/l ⩾ 6.0 kU/l.
App. K_m *L*-alanine	28 mmol/l	28 mmol/l	phosphate buffer pH 7.4, 81 mmol/l; NADH 0.16 mmol/l; 2-oxoglutarate 6.75 mmol/l; M_4 isoenzyme of lactate dehydrogenase 4.15 mg/l ⩾ 2.08 kU/l.
App. K_m 2-oxoglutarate	400 µmol/l	290 µmol/l	phosphate buffer pH 7.4, 81 mmol/l; *D*, *L*-alanine 65 mmol/l; NADH 0.16 mmol/l; M_4 isoenzyme of lactate dehydrogenase 4.15 mg/l ⩾ 2.08 kU/l.
App. K_m *L*-glutamate	10 mmol/l	12 mmol/l	triethanolamine pH 8.0, 87 mmol/l; pyruvate 2.12 mmol/l; NADH 0.2 mmol/l; ammonium acetate 106 mmol/l; glutamate dehydrogenase 333 mg/l ⩾ 30 kU/l.
App. K_m pyruvate	200 µmol/l	200 µmol/l	triethanolamine pH 8.0, 90 mmol/l; *L*-glutamate 50 mmol/l; NADH 0.2 mmol/l; ammonium acetate 106 mmol/l; glutamate dehydrogenase 67 mg/l ⩾ 6.0 kU/l.

optimal, according to l. c. (40). As in the assay of aspartate aminotransferase, we used a glutamate dehydrogenase-linked system for the opposite direction, and measured the production of 2-oxoglutarate.

The reaction mechanism of alanine aminotransferase is less studied than that of aspartate aminotransferase, but it seems to follow the same order (41) with similar inter-dependencies of optimum concentrations for the paired substrates. Thus for alanine aminotransferase the equality of the determined values is again a strong indication of the similarity of the catalytic sites of both preparations.

In contrast to aspartate aminotransferase there are no simple possibilities known for the removal of the co-

enzyme in either form from alanine aminotransferase preparations. Our experiments directed to the isolation of apoenzymes were also without success. Upon addition of pyridoxal phosphate to our purified alanine aminotransferase preparations we observed inactivation of both enzymes, depending to the same degree on the concentration of coenzyme added, and following the same time course.

Glutamate Dehydrogenase

The similarity of crystalline glutamate dehydrogenase preparations from human and beef liver in respect of pH-optima and apparent K_m-values as shown in table 7 is again sufficient. Deviations are at the most two-fold, which is not meaningful for practical use of the beef enzyme for replacement of human glutamate dehydrogenase in control materials.

The assay conditions chosen for the routinely used reaction in the direction of glutamate production were according to *E. Schmidt* (42). This method includes the activation of glutamate dehydrogenase by adenosine-5′-diphosphate, reported by *Frieden* (43). For the opposite direction of glutamate dehydrogenation we used arbitrary, suboptimal conditions to get possibly a better discrimination between the human and the animal enzyme. In the determination of the apparent K_m for NAD^+ we encountered problems with substrate inhibition.

The mechanism of the glutamate dehydrogenase has been extensively studied (for review see *Smith* et al. (44)). Substrates and coenzyme were shown to be bound in an at least partly random manner to the enzyme (45). The evaluation of true K_m-values (different ones for the different enzyme complexes) is further complicated by the allosteric properties of the enzyme (46).

Lehmann & Pfleiderer (23) isolated glutamate dehydrogenase from human liver in a slightly different way and compared their crystalline enzyme with our beef liver preparation. Their data on the human enzyme are very close to ours.

Lactate Dehydrogenase

Our data on the pH-optima and apparent K_m-values for the H_4- and the M_4-isoenzymes isolated from human and porcine material are shown in table 8. The agreement of the data obtained for the H_4-preparations is excellent. There are slight discrepancies for the M_4-isoenzymes from both sources, the human enzyme showing lower affinity for pyruvate, higher for lactate as compared to the porcine enzymes.

The assay conditions for pyruvate reduction are those of *Bergmeyer* et al. (47), for lactate dehydrogenation according to *Amador* et al. (48).

The mechanism of lactate dehydrogenase catalysis was shown to comprise a compulsory order of coenzyme and substrate binding to the enzyme (for review see l. c. (49)). The dissociation of the reacted coenzyme is rate limiting. Due to inhibitive and subunit-cooperative effects apparent K_m-values with this enzyme again are quite dependent on reaction conditions.

Comparitive studies on purified lactate dehydrogenase isoenzymes from different sources have been performed by several laboratories (e. g. *Pesce* et al. (50) and *Wachs-*

Tab. 7. Glutamate dehydrogenase from liver – Apparent K_m-values and pH optima – at 25 °C.

	Bovine liver	Human liver	Assay conditions (Concentrations of constituents in the assay solution) (8)
Optimum pH Substrate: 2-oxoglutarate	7.75	7.75	triethanolamine 31 mmol/l; ADP 1 mmol/l; EDTA 2.5 mmol/l; NADH 0.19 mmol/l; 2-oxoglutarate 6.4 mmol/l; ammonium acetate 100 mmol/l.
Optimum pH Substrate: glutamate	8.4	8.3	phosphate buffer 83 mmol/l; EDTA 3.3 mmol/l; NAD 0.5 mmol/l; *L*-glutamate 4.5 mmol/l.
App. K_m 2-oxoglutarate	5.0 mmol/l	2.9 mmol/l	triethanolamine pH 8.0, 31 mmol/l; EDTA 2.5 mmol/l; ADP 1 mmol/l; NADH 0.19 mmol/l; ammonium acetate 100 mmol/l.
App. K_m NADH	100 µmol/l	200 µmol/l	triethanolamine pH 8.0, 31 mmol/l; EDTA 2.5 mmol/l; ADP 1 mmol/l; ammonium acetate 100 mmol/l; 2-oxoglutarate 6.4 mmol/l.
App. K_m ammonia	16 mmol/l	16 mmol/l	triethanolamine pH 8.0, 31 mmol/l; EDTA 2.5 mmol/l; ADP 1 mmol/l; NADH 0.19 mmol/l; 2-oxoglutarate 6.4 mmol/l.
App. K_m glutamate	1.5 mmol/l	1.2 mmol/l	phosphate buffer pH 7.5, 83 mmol/l; EDTA 3.3 mmol/l; NAD 0.5 mmol/l.
App. K_m NAD	appr. 200 µmol/l product inhibition	appr. 100 µmol/l	phosphate pH 7.5, 83 mmol/l; EDTA 3.3 mmol/l; *L*-glutamate 4.5 mmol/l.

Tab. 8. Lactate dehydrogenase from heart and muscle (LDH-H_4 and LDH-M_4) – Apparent K_m-values and pH optima – at 25 °C.

	Human heart H_4 isoenzyme	Porcine heart H_4 isoenzyme	Human muscle M_4 isoenzyme	Porcine muscle M_4 isoenzyme	Assay conditions (Concentration of constitutents in the assay solution) (8)
Optimum pH Substrate: pyruvate	7.5	7.5	6.6–7.5	7.5	phosphate buffer 50 mmol/l; pyruvate 0.6 mmol/l; NADH 0.18 mmol/l.
Optimum pH Substrate: lactate	8.5–8.7	8.5–8.7	8.5–8.7	8.5–8.7	phosphate buffer 46 mmol/l; NAD 0.5 mmol/l; *L*-lactate 34 mmol/l.
App. K_m pyruvate	140 µmol/l	150 µmol/l	500 µmol/l	120 µmol/l	phosphate buffer pH 7.5, 50 mmol/l; NADH 0.18 mmol/l.
App. K_m NADH	10 µmol/l	11 µmol/l	11 µmol/l	12 µmol/l	phosphate buffer pH 7.5, 50 mmol/l; pyruvate 0.6 mmol/l.
App. K_m *L*-lactate	4.5 mmol/l	3.3 mmol/l	4.5 mmol/l	8.3 mmol/l	phosphate buffer pH 7.5, 46 mmol/l; NAD 0.5 mmol/l.
App. K_m NAD	63 µmol/l	67 µmol/l	110 µmol/l	100 µmol/l	phosphate buffer pH 7.5, 46 mmol/l; *L*-lactate 34 mmol/l.

muth & Pfleiderer (51). The latter group focussed their extensive studies on human isoenzymes from different organs (52, 53). Their overall results correspond to our data. *Emes* et al. (54) crystallized the H_4-isoenzyme from human heart muscle. Their apparent K_m-value for pyruvate of 102 µmol/l is in close agreement with ours.

Malate Dehydrogenase

Results on pH-optima and apparent K_m-values of mitochondrial malate dehydrogenase are reported in table 9. The enzyme preparations from human and porcine heart muscle are completely identical in these respects.

Malate dehydrogenase is only in minor use in Clinical Chemistry now. The conditions used for oxaloacetate reduction were according to *Bergmeyer* et al. (55); arbitrary conditions were used for malate dehydrogenation.

The mechanism of catalysis by malate dehydrogenase is of a similar compulsory order type to that of lactate dehydrogenase (56). The usefulness of apparent *Michaelis* constants is limited in the same way, but their close agreement under the same conditions again indicates a similarity between the catalytic sites of the human and porcine enzyme.

We have found no comparison data for human malate dehydrogenase nor on the purification of the enzyme from human heart. But the values found for the pig heart preparation in this study are in the same range as reported by *Grimm* (57).

Tab. 9. Malate dehydrogenase – Apparent K_m-values and pH optima – at 25 °C.

	Porcine heart	Human heart	Assay conditions (Concentration of constituents in the assay solution) (8)
Optimum pH Substrate: oxaloacetate	8.3	8.4	phosphate buffer 95 mmol/l; oxaloacetate 0.5 mmol/l; NADH 0.2 mmol/l.
Optimum pH Substrate: malate	10.0–10.5	10.0–10.5	glycine buffer 95 mmol/l; *L*-malate 120 mmol/l; NAD 4.4 mmol/l.
App. K_m oxaloacetate	33 µmol/l	33 µmol/l	phosphate buffer pH 8.3, 95 mmol/l; NADH 0.2 mmol/l.
App. K_m NADH	50 µmol/l	50 µmol/l	phosphate buffer pH 8.3, 95 mmol/l; oxaloacetate 0.5 mmol/l.
App. K_m *L*-malate	500 µmol/l	400 µmol/l	glycine pH 10.0, 95 mmol/l; NAD 4.4 mmol/l.
App. K_m NAD	250 µmol/l	250 µmol/l	glycine pH 10.0, 95 mmol/l; *L*-malate 120 mmol/l.

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